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EFFECTS OF UNMODIFIED AND CROSS-LINKED STROMA FREE HEMOGLOBIN SOLUTIONS ON BLOOD PRESSURE AND RENAL FUNCTION IN THE HYPOTENSIVE RAT

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We have examined the short term systemic and renal effects of stroma free hemoglobin in anesthetized rats following hemorrhagic hypotension. Two forms of SFH, one unmodified and the other modified by intramolecular crosslinking (o-raffinose polyhemoglobin) were compared. Both the unmodified and modified forms of SFH increased MAP in the hypotensive rats from 61±6 to 111±7 mm Hg and from 56±4 to 96± 5 mm Hg respectively. Furthermore, the reduction in GFR associated with hemorrhage was reversed by both

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SFH solutions. GFR increased in response to the unmodified SFH from 1.1±0.5 to 2.7±0.3 ml/min while the modified SFH increased GFR from 0.7±0.2 to 2.7±0.3 ml/min. In both SFH groups of rats the reduction in MAP and GFR were restored to prehemorrhage (baseline) values. While the hemodynamic effects of the two SFH solutions were comparable, urinary excretion rates of the unmodified SFH was about 100 fold higher (2.3±0.8 mg/min) than the modified cross-linked SFH (0.020±0.002 mg/min). The total amount of unmodified SFH excreted in the urine during the period of study represented more than 25% of the administered dose. In contrast, rats receiving the modified SFH excreted less than 0.5% of the administered dose over the period of observation. Finally, the fractional excretion of sodium was unaffected by infusion of the modified form of SFH. In contrast a pathological natriuresis was induced by the unmodified SFH; FeNa was increased approximately 20 fold above baseline by the unmodified SFH.

ABSTRACT

We have examined the short term systemic and renal effects of stroma free hemoglobin in anesthetized rats following hemorrhagic hypotension. Two forms of SFH, one unmodified and the other modified by intra-molecular crosslinking (o-raffinose polyhemoglobin) were compared. Both the unmodified and modified forms of SFH increased MAP in the hypotensive rats from 61±6 to 111±7mmHg and from 56±4 to 96±5mmHg respectively. Furthermore, the reduction in GFR associated with hemorrhage was reversed by both SFH solutions. GFR increased in response to the unmodified SFH from 1.1±0.5 to 2.7±0.3ml/min while the modified SFH increased GFR from 0.7±0.2 to 2.7±0.3 ml/min. In both SFH groups of rats the reduction in MAP and GFR were restored to prehemorrhage (baseline) values. While the hemodynamic effects of the two SFH solutions were comparable, urinary excretion rates of the unmodified SFH was about 100 fold higher (2.3±0.8mg/min) than the modified cross-linked SFH (0.020±0.002mg/min). The total amount of unmodified SFH excreted in the urine during the period of study represented more than 25% of the administered dose. In contrast, rats receiving the modified SFH excreted less than 0.5% of the administered dose over the period of observation. Finally, the fractional excretion of sodium was unaffected by infusion of the modified form of SFH. In contrast a pathological natriuresis was induced by the unmodified SFH; FeNa was increased approximately 20 fold above baseline by the unmodified SFH.

Key words: Blood substitute; cross-linked hemoglobin; o-raffinose polyhemoglobin, nephrotoxicity, blood pressure, hemorrhagic hypotension, renal function

INTRODUCTION

The availability of an acellular, stroma free hemoglobin (SFH) solution remains an important potential alternative to the use of blood transfusion (5). The original use of simple hemoglobin hemolysates were associated with a variety of serious side effects including kidney failure, intravascular coagulation, and anaphylactic reactions (1,20). However, it has become clear that removal of red cell stroma from the hemoglobin solutions reduces but does not prevent toxicity because the hemoglobin molecule itself has the potential to induce renal injury and dysfunction (3,8,20).

An understanding of the factors that mediate hemoglobin induced nephrotoxicity is important to facilitate the development of a non-toxic modified hemoglobin solution. It has been demonstrated in experimental models of intravascular hemolysis and rhabomyolysis that free intravascular hemoglobin and/or myoglobin can result in renal failure by a number of different mechanisms. The most widely recognized mechanism of hemoglobin induced renal failure is the nephronal obstruction that results from the intratubular precipitation of methemoglobin (22).

However, more recently free hemoglobin has been shown to have the potential to impair renal function in other ways. Hemoglobin can also induce renal insufficiency by causing intrarenal vasoconstriction (10). These hemodynamic effects of oxyhemoglobin have been demonstrated to be mediated, at least in part, by the inactivation of nitric oxide (NO) (11), a vasodilator constitutively produced by vascular endothelium which mediates vascular smooth muscle relaxation and plays an essential role in

the regulation of blood presssure and renal function (13). Furthermore, recent experimental evidence has also emerged indicating that hemoglobin or its metabolites cause direct renal cell injury by accelerating the production of reactive oxygen species (6,14).

During the past 10-15 years a substantial effort has been made to develop modified SFH solutions that avoid toxicity to the kidney and other organs while retaining the ability of the molecule to serve as an effective oxygen carrier. Most attention has been paid developing a modification that prevents the dissociation of the hemoglobin tetramer into dimers thereby minimizing the filtration of the hemoglobin at the glomerulus (5). These modifications, if effective should serve to prolong the intravascular retention time and therefore the efficacy of the administered SFH. Furthermore preventing dimerization of the hemoglobin molecule should also minimize filtration of hemoglobin at the glomerulus and the renal toxicity associated with the intratubular precipitation of metabolites of hemoglobin such as methemoglobin and hematin (22).

The nephrotoxic toxic effects of free intravascular hemaglobin have been found to occur predominantly in humans and animals that are volume depleted (8) . Since infusion of SFH is most likely to be necessary as a rescucitative fluid in volume depleted patients we have used a rat model of hemorrhagic hypotension developed in this laboratory (12) to compare short term effects of an human hemoglobin which is either unmodified or modified by cross-linking with o-raffinose (o-raffinose polyhemoglobin (Hemosafe®)) (U.S. Patent No. 4,857,636 (1989)).

METHODS

Male Sprague-Dawley rats, weighing between 250-350g were used for all experiments. Rats were fed regular Purina Rat Chow (Purina Mills, Chicago, IL) and allowed free access to water. Anesthesia was induced with an intraperitoneal injection of pentobarbitol sodium (5 mg/100 gm body wt) and then maintained with a constant intravenous infusion of pentobarbitol (91 mg/min) throughout the study. Rats were placed on a thermostatically controlled heated table and body temperature was monitored with a rectal thermometer and maintained between 36 and 38°C. A tracheotomy was formed with the use of polyethylene (PE-240) tubing and the femoral artery was cannulated with PE-50 tubing for blood pressure monitoring as well as blood sampling. The left internal jugular vein was cannulated with two catheters of PE-50 tubing. A bladder catheter (PE-90) was placed by a suprapubic incision for urine sampling. GFR was measured by determining the clearance of methoxy-{^{3}H} inulin (New England Nuclear, Boston, MA). Inulin and pentobarbitol sodium dissolved in 5% dextrose water was infused into one venous catheter at a rate of 0.028ml/min while the other internal jugular catheter was used for the infusion of either the control solution (albumin) or one of the two forms of stroma free hemoglobin.

Experimental Protocols

After an equilibration period of 30 minutes, two 20 minute control clearance periods were obtained during which blood pressure was measured continuously and urine collected for the measurement of inulin clearance, urine flow rate and sodium excretion. At the end of this period

the rats were subjected to hemorrhage. Whole blood (20 ml/kg body wt) was removed through the femoral arterial catheter at the rate of one ml per minute. After a further 45 minute equilibration period, a single second 20 minute clearance ("hemorrhage" period), was obtained for the measurement of blood pressure inulin clearance, urine flow rate and sodium excretion.

Then, the rats were randomly divided into two experimental groups that received either the unmodified (n=8) or modified (n=7) SFH solution. Each experimental group was compared to two separate control groups that received albumin solutions with oncotic pressures that matched the two SFH solutions (n=8 in each control group) (Table 1).

Following a further equilibration period of 15 minutes, two 20 minute ("post-infusion") clearance periods were obtained. At the end of the experiment, blood was rapidly obtained from the abdominal aorta from group 2 and 3 rats for measurement of total plasma hemoglobin.

Human hemoglobin was modified by crosslinking with raffinose-(0-raffinose polyhemaglobin, U.S. Patent No. 4,857,636 (1989)) provided by Hemosol Inc. (Etobicoke, Ontario, Canada) Unmodified SFH was human hemoglobin that was purified by ultrafiltration without further modification. Both the albumin and SFH solutions were administered dissolved in 2ml of Ringers Lactate which was infused over 10 minutes. The concentrations and oncotic pressures of the two albumin control solutions, the unmodified hemoglobin and the cross-linked hemoglobin are provided in Table 1. The concentration of the modified hemoglobin preparation was higher than the unmodified hemoglobin preparation (Table 1). As a result, the rats receiving unmodified SFH received a total dose of 346 mgs while the rats receiving the modified hemaglobin received a

lower total dose of 234 mgs. The characteristics of the two SFH solutions are provided in Table 2.

Analytical Methods

Concentrations of methoxy-{3H} inulin in urine and plasma were determined by liquid scintillation counting. Urine sodium concentrations were measured by flame photometry. Inulin clearances (GFR) were calculated with standard formulas. The two clearance periods obtained during the control and post-infusion periods were meaned.

A blood gas analyser (Instrumentation Laboratories, Model 3813, . (Waltham MA.) was used to measure the p02, pCO2 and pH of the SFH solutions. Total hemoglobin was measured as previously described (10). Oxy- and methemoglobin were measured with a co-oximeter (Instrumentation Laboratories, Model #282, Waltham MA.). The p50 of the hemoglobin preparations was measured with a Hemoxanalyser (2).

Abbreviations.

SFH= stroma free hemoglobin

MAP=mean arterial pressure

GFR=glomerular filtration rate

FeNa=fractional excretion of sodium

Statistics

All data are presented as the means ± SEM. All comparisons within each group (baseline, hemorrhage and post-infusion periods) were made using analysis of variance (ANOVA) followed by the Scheffe test. Statistics were calculated on a Macintosh computor using the Statworks® software program. A p value of <0.05 was considered significant.

RESULTS

Hemorrhage resulted in a comparable fall in blood pressure as well GFR in all groups . The administration of both the unmodified and the modified (o-raffinose polyhemoglobin) SFH solutions to the hypotensive rats resulted in a rise in MAP as well as GFR to levels comparable to baseline. The two albumin solutions also increased MAP and GFR. However, neither albumin control solution increased these values to the same extent as the SFH solutions; in both control groups the MAP and GFR remained substantially below baseline levels after the albumin solutions were infused (Tables 1 and 2; Figures 1 and 2).

Hemorrhage did not alter the urine flow rate or FeNa in any of the three groups studied (Tables 3 and 4). Similarly, the infusion of the albumin solutions (Tables 3 and 4) as well as the modified SFH (Table 4) did not alter FeNa. In contrast the unmodified SFH resulted in a substantial diuresis and increase in FeNa. (Table 3).

The unmodified SFH was rapidly excreted in the urine at a rate of 2.3±0.8 mg/minute while the cross-linked hemoglobin was excreted in very small amounts (0.02 ± 0.002 mg/min) (Figure 3).

The concentrations of total hemoglobin measured in blood samples obtained at the end of the experiment were no different between rats receiving the unmodified SFH (1.7±0.3g%) and and those receiving the cross-linked SFH (1.6±0.1g%).

DISCUSSION

The administration of SFH following hypotension induced by hemorrhage represents a relevant model to test the potential benefits and side effects of these agents (7). We have compared the short term, systemic and renal effects of modified and unmodified forms of SFH using albumin solutions with comparable colloid osmotic pressures as controls (Table 1). The control solutions would therefore be expected to produce approximately the same degree of intravascular volume expansion induced by osmotic shifts of extravascular fluid into the intravascular space. As expected, both albumin solutions resulted in increases in both blood pressure and GFR, (Tables 1 and 2), effects likely due to intravascular volume expansion.

Both unmodified and modified solutions of SFH increased MAP to values comparable to the baseline, pre-hemorrhage values. While the albumin solutions also increased blood pressure, the effect of these control solutions was significantly less than that of the SFH solutions (Figures 1 and 2). Thus the SFH solutions both had a hypertensive effect that was independent of the colloid activity of these solutions.

There is substantial evidence that hemoglobin increases systemic blood pressure in normotensive animals (1) and in humans (21). Recently, hemoglobin has been also been reported to cause a marked increase in blood pressure in swine following hemorrhagic hypotension (7), a result comparable to that reported in this study. The hypertension induced by hemoglobin has been demonstrated to be due to systemic vasoconstriction (7).

Since oxyhemoglobin binds to and inactivates NO, it has been postulated that the vasoconstrictor effect of SFH is due, at least in part, to reduced availability of NO (11,13). Studies examining the effects of competitive inhibitors of NO production have demonstrated that NO inhibition causes marked vasoconstriction and hypertension in experimental animals (9,12,15). While hemoglobin reduces NO activity by inactivating the NO molecule rather than by inhibiting its production, the functional result i.e. reduced availability of NO and systemic vasoconstriction is comparable (7,11).

A novel and unexpected finding of this study was the substantial improvement in GFR induced by SFH in the hypotensive rats. Both modified and unmodified solutions reversed the functional renal failure caused by the low renal perfusion pressure. If we postulate that the hypertensive effect of SFH is mediated by the inactivation of NO, this response of GFR to SFH seems paradoxical, since a number of investigators have clearly demonstrated that inhibition of NO production causes intrarenal vasoconstriction and decreases both renal plasma flow and GFR in normotensive animals (9,15,17).

However, we have evidence to support the hypothesis that the beneficial effect of SFH solutions on GFR demonstrated in our hypotensive rats can also be explained by reduced availability of NO. This hypothesis is based on previous observations reported by our group that NO inhibition produces opposite effects on GFR depending on whether inhibitor of NO is given to a normotensive or hypotensive animal (12). While NO inhibitors reduce GFR in the normotensive animals (9,15,17) the same intervention markedly improves renal function in rats subjected to hypotensive hemorrhage (12), a result similar to that demonstrated in response to SFH

in this study. The mechanism responsible for our observation that NO inhibition improves GFR in hypotensive rat remains to be fully elucidated. Thus, we hypothesize that the hypertensive effect of SFH that has been demonstrated by many investigators in normotensive animals, and that has also been reported by Hess et al (7) and by our group in this report is mediated, at least in part, by oxyhemoglobin-mediated inactivation of constitutively produced NO.

We have also demonstrated that while only small amounts of the modified hemoglobin was excreted in the urine, there was substantial loss of the unmodified form of SFH in the urine. The rate of unmodified SFH was approximately 100 fold that of the modified SFH. A total of approximately 92 mg of unmodified SFH was excreted during a 40 minute period representing about 25% of the total dose of unmodified hemoglobin (346 mg) that was infused. In contrast, only 0.8mg of modified SFH was excreted in the urine during the same time period, an amount that represents less than 0.5% of the total dose given (234mg). The loss of unmodified SFH in the urine likely explains the observation that while the total dose of unmodified SFH administered was subtantially larger than that of modified stroma free hemoglobin the final concentration of SFH in the plasma measured at the end of the experiment was comparable between the two groups. Thus, the method used to polymerize the modified hemoglobin tested in this study was clearly effective in minimizing urinary excretion of the molecule.

While the modified and unmodified forms of SFH produced comparable effects on MAP and GFR, the effects of these two preparations on urine flow rate and sodium excretion was strikingly different. While neither the albumin vehicle or the modified, cross-linked form of SFH

resulted in any change in either of these variables, the infusion of the unmodified form of hemoglobin was associated with a profound increase in urine flow rat and sodium excretion. (Table 3). Similar effects on sodium and water handling by the kidney has beem reported in response to unmodified SFH by other investigators (7). While the profound natriuresis induced by unmodified SFH probably represents a direct toxic effect of the filtered hemoglobin on tubular function, the mechanism responsible for this effect was not determined in this study. Interestingly, hemoglobin has been shown to inhibit Na/K ATPase activity in neuronal cells (16). Further studies are necessary to elucidate the mechanism/s by which hemoglobin or its metabolites may derange the normal transport function of renal tubular cells.

It is important to emphasize that while this study was useful in examining the short term hemodynamic and renal effects of SFH in the hypotensive animal, relatively low plasma concentrations of SFH (1.6-1.7g/100ml) were achieved. The effects of much higher, therapeutically useful concentrations need to be examined. Furthermore, longer term studies are necessary to exclude toxic effects of SFH solutions. The degradation products of hemoglobin that are particularly toxic to renal cells have been shown to accumulate in the kidney days after administration of modified SFH solutions and may result in delayed nephrotoxicity (4,19). Also, while the hemodynamic effects of the SFH solutions resulted in an improvement in GFR in this study, the vasoconstrictive effects of hemoglobin clearly have the potential to cause deleterious effects. The systemic vasoconstriction induced by SFH has been shown to prevent the the major anticipated benefit of this therapy, i.e. improvement in oxygen delivery to tissues in one recent study (7).

Other studies have shown that SFH induces both pulmonary (7) and coronary vasoconstriction (18), effects that may have harmful effects on pulmonary and/or cardiac function . For all these reasons, additional studies are necessary to determine whether any particular form of modified SFH administered in large amounts is beneficial and free of serious side effects.

In summary, we have reported that both modified and unmodified forms of hemoglobin result in marked improvement in blood pressure as well as GFR in severely hypotensive rats with functional renal failure. The unmodified SFH is filtered at the glomerulus and induces a pathologiocal natriuresis. In contrast, the modified SFH is filtered and excreted in extremely small amounts, indicating successful cross linking of the tetrameric molecule in this particular preparation.

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Table 1

Comparison of oncotic pressures of the solutions of unmodified and modified SFH and the albumin (control) solutions.

	Concentration (g/dL)	Oncotic pressure (mmHg)
	UNMODIFIED SFH	
SFH solution	17.3	92.4
Control albumin solution	16.8	94.0
	MODIFIED SFH	
SFH solution	11.7	21.0
Control albumin solution	5.0	21.0

Table 2
Characteristics of the unmodified and modified (o-raffinose cross-linked) stroma free hemoglobin (SFH) solutions

	Unmodified SFH	Cross-linked SFH
Hemoglobin concentration(g/dl):	17.3	11.7
O_2 Hemoglobin (%):	96.7	86.6
Co Hemoglobin (%):	3.5	2.5
Methemoglobin (%):	0.2	6.6
Volume O_2 (%):	23.2	14.0
p50 (Torr at pH 7.4):	14.0	22.2

Table 3

Effect of the albumin vehicle (control group) and the unmodified SFH on mean arterial pressure (MAP), glomerular filtration rate (GFR) and fractional sodium excretion (FeNa) following hemorrhage

	BASELINE PERIOD	HEMORRHAGE PERIOD	POST-INFUSION PERIOD
MAP (mmHg)			
Control Group	114 ± 2	63 ± 3*	82 ± 2*†
Unmodified SFH	110 ± 5	61 ± 6*	111 ± 7†
GFR (ml/min)			
Control Group	3.0 ± 0.2	1.0 ± 0.2*	2.2 ± 0.2*†
Unmodified SFH	2.8 ± 0.3	1.1 ± 0.5*	2.7 ± 0.3†
Urine flow rate (ul/min)			
Control group	13.5 ± 7.0	3.0 ± 0.4	11.0 ± 2.0
Unmodified SFH	8.3 ± 1.0	5.0 ± 2.0	81 ± 11*†
FeNa (%)			
Control Group	0.08 ± 0.02	0.08 ± 0.04	0.06 ± 0.02
Unmodified SFH	0.10 ± 0.04	0.06 ± 0.03	2.10 ± 0.60*†

*=$p<0.05$ vs. baseline period
†=$p<0.05$ vs. hemorrhage period

Table 4

Effect of the albumin vehicle (control group), and the modified SFH on mean arterial pressure (MAP), glomerular filtration rate (GFR) and fractional sodium excretion (FeNa) following hemorrhage

	BASELINE PERIOD	HEMORRHAGE PERIOD	POST-INFUSION PERIOD
MAP (mmHg)			
Control Group	109 ± 2	59 ± 4*	71 ± 5*†
Modified SFH	105 ± 4	56 ± 4*	96 ± 5†
GFR (ml/min)			
Control Group	3.1 ± 0.3	1.0 ± 0.3*	1.9 ± 0.2*†
Modified SFH	2.5 ± 0.2	0.7 ± 0.2*	2.7 ± 0.3†
Urine flow rate (ul/min)			
Control group	7.8 ± 0.6	6.1 ± 1.7	8.5 ± 0.9
Modified SFH	7.2 ± 0.7	4.0 ± 1.4	9.4 ± 3.6
FeNa (%)			
Control Group	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.02
Modified SFH	0.07 ± 0.02	0.06 ± 0.02	0.08 ± 0.04

*=$p<0.05$ vs. baseline period

†=$p<0.05$ vs. hemorrhage period

LEGENDS

Figure 1

Effects of the unmodified SFH (cross-hatched bar) and the iso-oncotic albumin control solution (shaded bar) on mean arterial pressure (upper panel) and glomerular filtration rate (lower panel) following hemorrhagic hypotension

*=$p<0.05$ compared to control period within same group

†=$p<0.05$ compared to the hemorrhage period within the same group

(n=8 in both groups)

Figure 2

Effects of the modified (cross-linked) SFH (cross hatched bar) and the iso-oncotic albumin control solution (shaded bar) on mean arterial pressure (upper panel) and glomerular filtration rate (lower panel) following hemorrhagic hypotension

*=$p<0.05$ compared to control period within same group

†=$p<0.05$ compared to the hemorrhage period within the same group

(n=8 in control; n=7 in modified SFH group)

Figure 3

Urinary excretion rate of unmodified and modified forms of SFH

*p=<0.05 compared to unmodified SFH

Figure 1

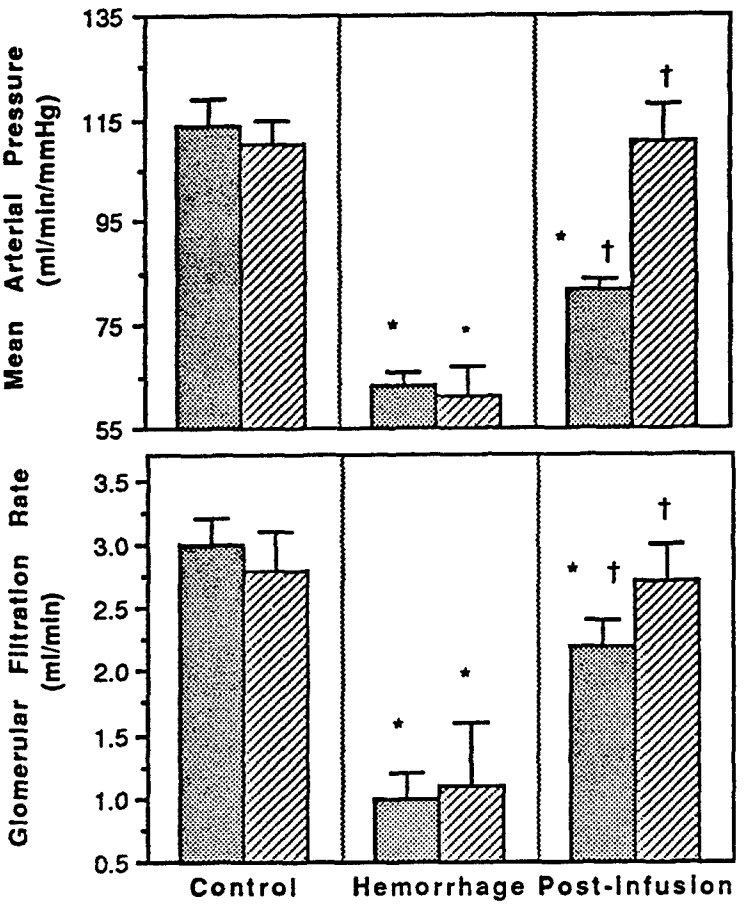

Mean Arterial Pressure (ml/min/mmHg)
135
115
95
75
55
Glomerular Filtration Rate (ml/min)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Control
Hemorrhage
Post-Infusion

Figure 2

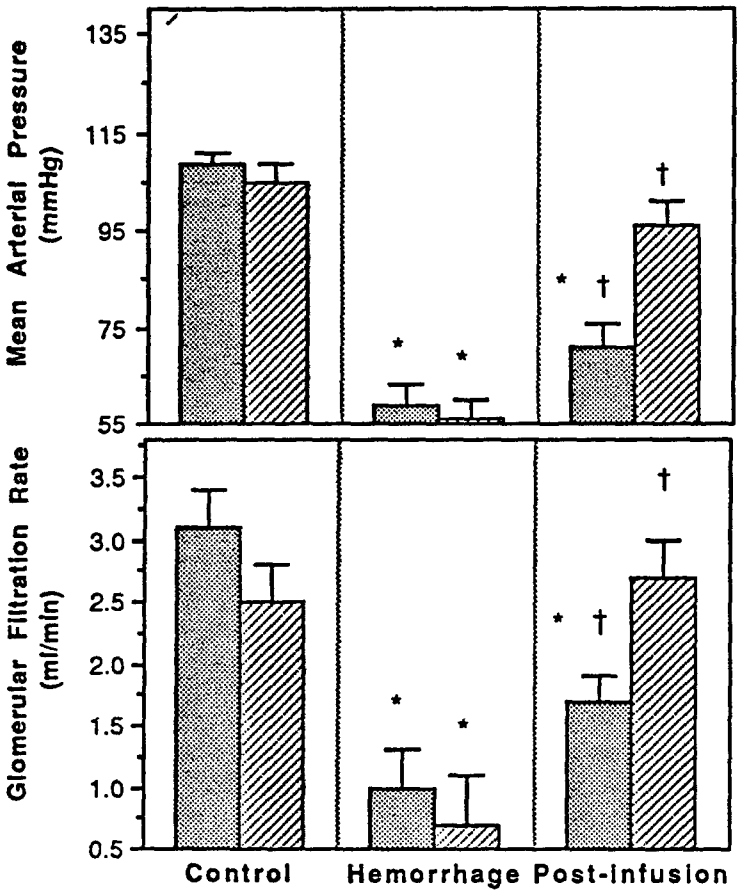
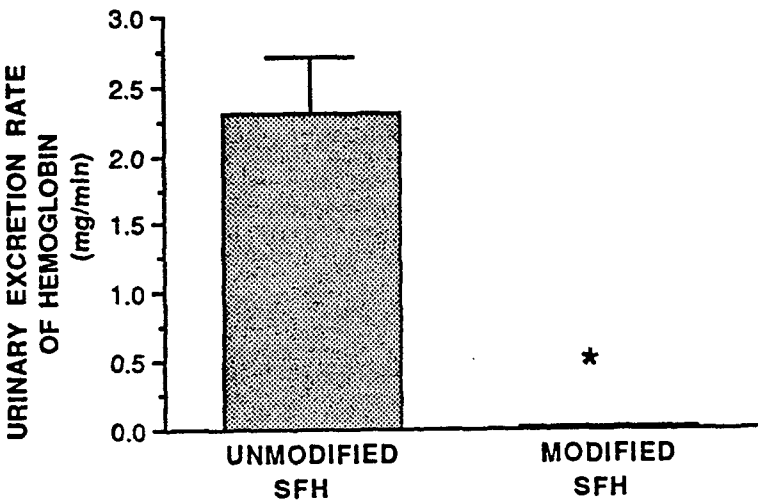

Mean Arterial Pressure (mmHg)
135
115
95
75
55
Glomerular Filtration Rate (ml/min)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
*
†
Control
Hemorrhage
Post-Infusion

Figure 3


URINARY EXCRETION RATE
OF HEMOGLOBIN
(mg/min)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
UNMODIFIED
SFH
MODIFIED
SFH